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### Cancer formation and the spindle assembly checkpoint

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**CANCER FORMATION AND THE  
SPINDLE ASSEMBLY CHECKPOINT**

A Senior Honors Project in Partial Fulfillment of  
Bachelor of Science with University Honors in  
Biological Sciences: Biochemistry and Cellular and  
Molecular Biology  
The University of Tennessee, Knoxville

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April 2006

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### **Abstract**

The purpose of this project is to generate double mutants for *p53* and *BubR1* in a BALB/c background that is known to have an increased susceptibility to mammary tumors. Our hopes are that the two mutations in a BALB/c background would create a mouse model susceptible to mammary gland tissue tumorigenesis. *P53* and *BubR1* mutants are characterized by high tumor susceptibility due to dysfunctions in the cell cycle checkpoints G1/S and the spindle assembly checkpoint.

Our initial analysis indicates that we have created double mutants in an 87.5% pure BALB/c background. We have not yet observed cancer in the mammary tissue; however, we have seen other cancer formations and abnormalities in the colon that may be precancerous. Our future goals are to backcross our BALB/c double mutants a total of ten times (N10) to achieve a pure BALB/c background. We will continue to monitor the colony for tumor formation, specifically in the mammary tissue. We also hope to see the embryonic lethality of *BubR1* nullizygous mutants rescued in a pure BALB/c and nullizygous *p53* background.

## **Introduction**

Cancer is the uncontrolled proliferation of cells that occurs when mistakes in DNA repair and replication lead to dysfunctional cell cycle checkpoints. The cell cycle is the necessary and ubiquitous process that allows all organisms to survive and grow. Because the cell cycle is such an important function for the proliferation of cells, it is regulated by three important checkpoints. These checkpoints occur to chronologically check for DNA damage, accurate DNA replication and proper spindle alignment before chromosomal division; this activity ensures that genetic integrity is maintained during the equal distribution of genomic content to the daughter cells.

## **The Cell Cycle**

The genetic material of an organism is found on its DNA which is packed into chromosomes. Cellular division requires the accurate replication and correct segregation of identical chromosomes into two daughter cells. The goal of the organism (at a cellular level) is to form two duplicate daughter cells, and the cell cycle is the means by which this is achieved.

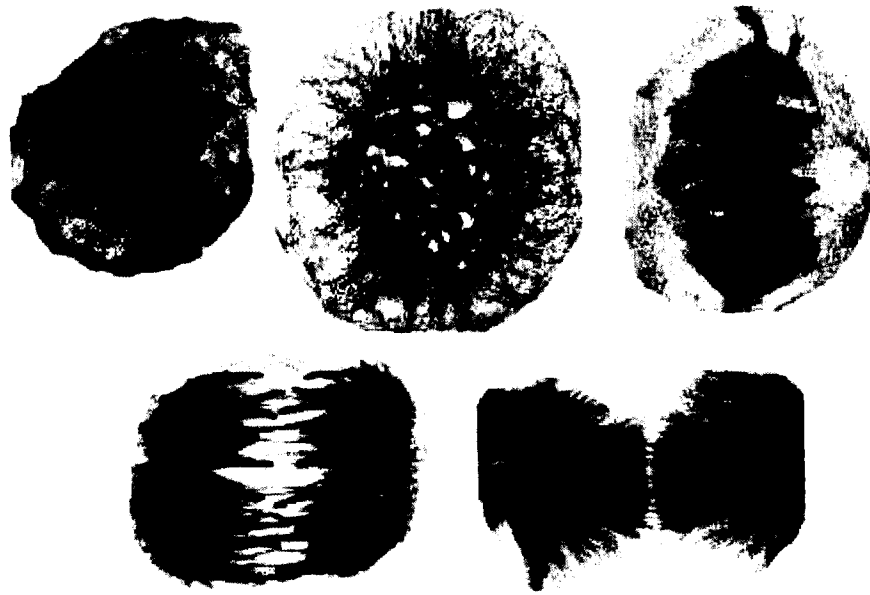
The cell cycle is comprised of four different phases, G1, S, G2 and mitosis. G1 is the first stage a cell enters

upon completing cytokinesis, or the division of cytoplasm between daughter cells. The G1 phase can last from a few hours to a few years. When a cell stops dividing or is inactive, this is known as G0. The G1 phase is the point at which the cell actively grows and produces RNA and the proper proteins it needs to begin DNA synthesis.

The next step in the cell cycle is DNA replication which occurs during the S phase. DNA replication results in the production of sister chromatids which contain exact copies of the cell's genetic information. Sister chromatids are linked at the centromere. It is here at the centromere that a protein structure known as the kinetochore forms. This structure serves as a point at which microtubules attach during mitosis to separate the sister chromatids into their respective daughter cells. Before this segregation can happen, however, the cell must go through the G2 phase in which it doubles in size and continues to produce proteins necessary for the continuation of the cell cycle.

Mitosis is the final phase of the cell cycle and is the phase in which cellular division actually occurs. Mitosis will ideally result in two identical progeny and is made up of five stages: prophase, prometaphase, metaphase, anaphase, and telophase. Prophase, the initial stage of

mitosis, is characterized by chromosome condensation and the formation of mitotic spindle, a network of microtubules. The prometaphase stage follows in which the nuclear membrane disintegrates and allows the microtubules to attach at the kinetochores of the sister chromatids. The chromatids then begin to migrate to the mid line of the cell known as the metaphase plate. Metaphase is marked by the complete alignment of the chromatids along the mid-line of the cell. The sister chromatids are attached at the kinetochore which is joined with microtubules from opposite poles of the cell. Anaphase begins if there is proper alignment of the chromatids and attachment to microtubules. Sister chromatids separate at their kinetochores during anaphase and are pulled towards opposite poles of the cell by the microtubules. Telophase then marks the end of mitosis. In this stage, the nuclear membrane reforms and cellular division is complete upon cytokinesis. If the cell cycle works properly, the result is two indistinguishable daughter cells.



**Figure 1.** Microscopic photographs showing each of the five stages of mitosis. (Image from <http://tidepool.st.usm.edu/crswr/mitosis.html>).

### **Cell Cycle Checkpoints**

In order to ensure that DNA is replicated without mistakes and the sister chromatids are properly lined along the metaphase plate and separated equally into each daughter cell, the cell cycle has three checkpoints. The first checkpoint is known as G1/S and occurs as the transition between the G1 and S phases. This checkpoint is activated by DNA damage and prevents replication until the damage is fixed, or, if the mistakes are irreparable, it causes the cell to go through apoptosis or cell death. The next checkpoint occurs at the transition between G2 and mitosis. This cell cycle arrest allows the cell to check

the DNA template and the newly replicated DNA for any damages or mistakes before the cell moves on to divide and distribute its DNA. The final checkpoint of the cell is the mitotic spindle checkpoint. It is here that microtubules from opposite poles of the cell are checked to be properly attached to the sister chromatids on the equatorial plate so that tension is generated across the kinetochore.

### **Important Checkpoint Proteins**

**p53:** Because cancer is a disease characterized by the uncontrolled proliferation of cells, it is important to study the mechanisms of the cell cycle to uncover important genes and proteins that are responsible for proper checkpoint function. Mutations in these genes or loss of a functioning protein may lead to a loss of cell cycle control and, ultimately, a cancer phenotype. One of the most observed mutations in human cancers occurs in the p53 gene. P53 is a checkpoint protein that is active in both the G1/S and G2/M checkpoints. This prevalence of p53 mutations makes the study of its function in the cell cycle of utmost importance in understanding cancer and its possible treatment therapies.

P53 is a tumor suppressor that is capable of inducing a great number of genes in response to DNA damage and toxic



insult to the genome. The induced genes serve to arrest the cell cycle, induce the transcription of proteins that negatively regulate p53 levels, or promote apoptosis in the event of irreparable DNA insult. P53 is activated by upstream ATM/ATR kinases and the activated form of p53 signals the cell to arrest or die. The consequences of p53 activation protect the cells DNA from accumulating errors by continuing to divide with damaged DNA.

As explained above, p53 serves an obviously important role in maintaining genomic integrity by controlling the induction of cell cycle checkpoints or, in the worst cases, apoptosis. It is no surprise then that p53 mutations or loss are so often seen in cancer because they prevent p53 from activating cell cycle arrest or apoptosis (Goodsell, 1999). Without p53, the cell continues to divide, unchecked, with damaged or broken DNA, and these mutations perpetuate the occurrence of yet more mistakes during the course of several cell divisions (Ljungman, 2000).

**BubR1:** The BubR1 protein plays a major role in the mitotic spindle checkpoint. It ensures the proper attachment of microtubules to the kinetochores prior to anaphase and the correct, subsequent segregation of sister chromatids. BubR1 is, therefore, exceedingly important in the prevention of chromosomal instability (CIN). As could be

expected, mutated forms of BubR1 have been seen in cancer phenotypes, specifically colon cancer; however *BubR1* deletion appears to be rare (Cahill, 1998).

The exact function of BubR1 is quite complex, and though many of its roles have been uncovered, there is still a great deal of information to gather on the full function of this protein in the cell cycle. It has been proven that BubR1 protein levels peak during the G2/M phase of the cell cycle, which is evidence for its function in the spindle assembly checkpoint (Davenport, 1999). BubR1 has been shown to interact with the centromeric motor protein E (CENP-E), and it localizes to the kinetochores of unaligned chromosomes followed by the localization of CENP-E (Chan, 1998). BubR1 prevents mitotic exit until all the chromosomes are properly aligned, and one of its main roles is to sense tension across the kinetochore, which signals microtubule attachment (Skoufias, 2001). When BubR1 is activated through phosphorylation facilitated by Mad1, it interacts with Cdc20, the cofactor that binds the Anaphase Promoting Complex (APC) and allows the cell to continue through anaphase. BubR1s interaction with Cdc20 effectively arrests the cell during mitosis so that adequate spindle attachment and chromosome separation may occur (H. Yu, 2002).

Since BubR1 is so important to the spindle checkpoint and in avoiding CIN, its failure or a loss of the gene itself could result in a severe threat to genomic instability and possible aneuploidy or polyploidy, characteristics often seen in cancer cells. In order to determine the necessity of *BubR1*, knock-out mice have been created using a gene trap. The BubR1 haploinsufficient mice were then bred to examine the effects of the loss of one or both *BubR1* alleles. It was determined that *BubR1* is an essential gene because nullizygous embryos began dying at 6.5 days post coitum (dpc) and were reabsorbed by 8.5 dpc (Wang, 2004). Death was due to apoptosis, and BubR1 was determined to be an essential protein for embryonic development and the monitoring of spindle assembly during mitosis.

**Rationale for Generating BubR1/p53 Double Mutants in a**

**BALB/c background:** Here, we have studied the synergistic effects of p53 deletions and BubR1 deficiency in mice. Several cancer studies have shown that several mutations or deletions of genes involved in cell proliferation are required to obtain a cancer phenotype. It has already been observed that p53 is often seen deleted in colon cancer phenotypes (Cahill, 1998). We hypothesize that making p53 heterozygous or nullizygous mice also deficient for BubR1

will increase the tumor susceptibility of the mice and, likewise, decrease the lifespan; it is also possible that we will observe colon cancer phenotypes. The compound mutants were generated and frequent health checks were performed to watch for the development of cancer in the mutant mice.

Our double mutants for p53 and BubR1 have shown to have the expected increased susceptibility to tumorigenesis. The next step in the project was to generate these deficient mice into another cancer susceptible background known as BALB/c. BALB/c mice have strain-specific polymorphisms in their DNA-dependent protein kinase (*prkdc*) which makes them less able to repair DNA damage such as double strand breaks (DSB) (Y. Yu, 2001). It has been determined that post-irradiation damage has a much higher rate of repair in the C57BL/6 strain (our laboratory's common mouse strain) than in BALB/c mice (Y. YU, 2001). The amount of *prkdc* in BALB/c mice is significantly reduced, which is important because *prkdc* is actively involved in end-rejoining of DSB, apoptosis and signal transduction in the cell (Y. Yu, 2001 and Smith, 1999). Genomic instability has thus far been seen specifically in mammary epithelia of female mice (Y. Yu, 2001). Our goal was to place the already cancer

susceptible p53/BubR1 double mutant into the BALB/c background in hopes of seeing a mammary gland cancer phenotype in our female mice.

### **Materials and Methods**

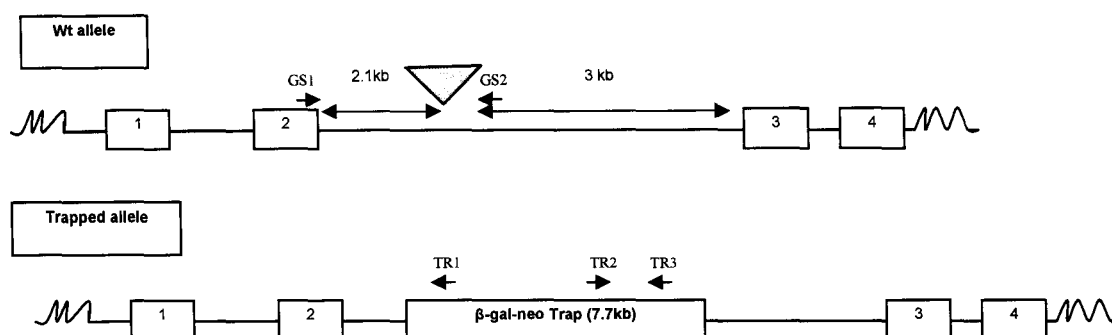
**Generation of BubR1/p53 mice in BALB/c background:** In order to generate BubR1 deficient mice, a gene trap approach was used to disrupt the *BubR1* gene. We obtained embryonic stem (ES) cells with a gene trap inserted into *BubR1* from Bay Genomics. The trap was shown to have inserted into intron 2 of *BubR1*. This was confirmed by PCR and Southern blotting. When the insertion was confirmed, the trapped cells were injected into 3.5 dpc blastocysts and inserted into a pseudo-pregnant C57BL/6 female mouse. Nine pups were generated, four of which produced germ line litters for the *BubR1* deletion. Tails tips were cut and taken for genetic analysis, which indicated equal numbers of wild type and heterozygous offspring. To generate p53/BubR1 double mutants, the BubR1 mice were mated to our p53 knockout strains.

Once the double mutant colony was established, we obtained BALB/c mice from Jackson Laboratories. Mice heterozygous or nullizygous for p53 and heterozygous for BubR1 were mated with the BALB/c females to begin producing double mutant offspring in a BALB/c background.

**DNA Extraction:** To isolate DNA for our genotyping experiments, we clip the tails of our mice when we wean them. We then incubate the tail overnight at 55°C in 500µL tail lysis buffer (50mM Tris pH 7.5, 50mM EDTA, 100mM NaCl, and 1% SDS), 10µL 10ng/µL Proteinase K and 1.25µL 2M DTT. After incubation, 500µL phenol chloroform is added to the tails and mixed by hand. The samples are then microcentrifuged for two minutes at 13,000rpm. Approximately 400µL of the top, aqueous layer is isolated and placed in a second tube. The bottom layer is discarded. To this second tube, 800µL 100% ethanol is added and the samples are mixed gently with the hand until a precipitate is formed. This precipitate is the DNA and is removed from the ethanol and placed into a final tube with 100µL 5mM Tris. The DNA is allowed to equilibrate overnight at room temperature before being used for genotyping.

**BubR1 Genotyping:** Polymerase chain reaction (PCR) was used to genotype BubR1 mice. For each reaction, 200ng of DNA is added to a 23µL PCR master mix composed of 1X PCR buffer, 1.5mM MgCl<sub>2</sub>, 1.5µL of dNTP, 0.2µM of forward and reverse primers and 1.0 unit of Platinum Taq DNA Polymerase. For the insertion and wild type PCR, the conditions are as follows: a two minute denaturation step at 94°C precedes

another denaturation step for 30 seconds at 94°C, annealing for 30 seconds at 54°C, and finally extension for two minutes and fifteen seconds at 72°C. This is repeated for 32 complete cycles and is finalized by a five minute extension step at 72°C. The trap PCR conditions are similar except for a decrease in extension time (one minute and fifteen seconds). The primers for the trap PCR are 1300f and 2000rr which create a product about 665 base pairs (bp) in length. The primers used to verify the correct insertion of the trap are Bub2e2s and 984r, which gives a PCR product of 2.4kbp. The two products are proof of the presence and correct insertion (location) of the gene trap in *BubR1*. To determine the presence of the wild type allele, primers Bub2e2s and Bub2in2a are used and give a PCR product 2.25kbp in length. This product indicates the presence of an uninterrupted *BubR1* gene, specifically in its second intron.



**Figure 2.** Schematic showing the trap insert into the *bubR1* gene. TR2 and TR3 represent the primers used to determine the presence of the trap. GS1 and TR1 represent the primers to determine if the trap was inserted into the correct place in the genome. GS1 and GS2 represent the primers used to characterize the wild type allele.

**P53 Genotyping:** The p53 genotype of mice is determined by Southern blot assays. Two and one half micrograms of DNA is digested with BamH1 overnight at 37°C. The samples are then loaded into a 0.8% agarose gel containing ethidium bromide. Once the gel is run, the DNA is denatured with 0.25N HCl and transferred onto a nylon membrane overnight in 0.4N NaOH. Once the gel is transferred, the membrane is rinsed in 2X SSC and incubated at 37°C for one hour to dry. The membrane is then placed into a hybridization cylinder with 30mL of pre-hybridization buffer (1.3% SDS, 2X SSPE, 1% milk, and 2mg denatured salmon sperm DNA) for five to six hours at 68°C. The pre-hybridization buffer is then removed and hybridization buffer (10% dextran sulfate, 1.5X SSPE, 1% SDS, and 0.5% milk) is added along with a radio-labeled DNA probe (1,000,000cpm/mL hybridization buffer). The membrane is incubated in the radio-labeled



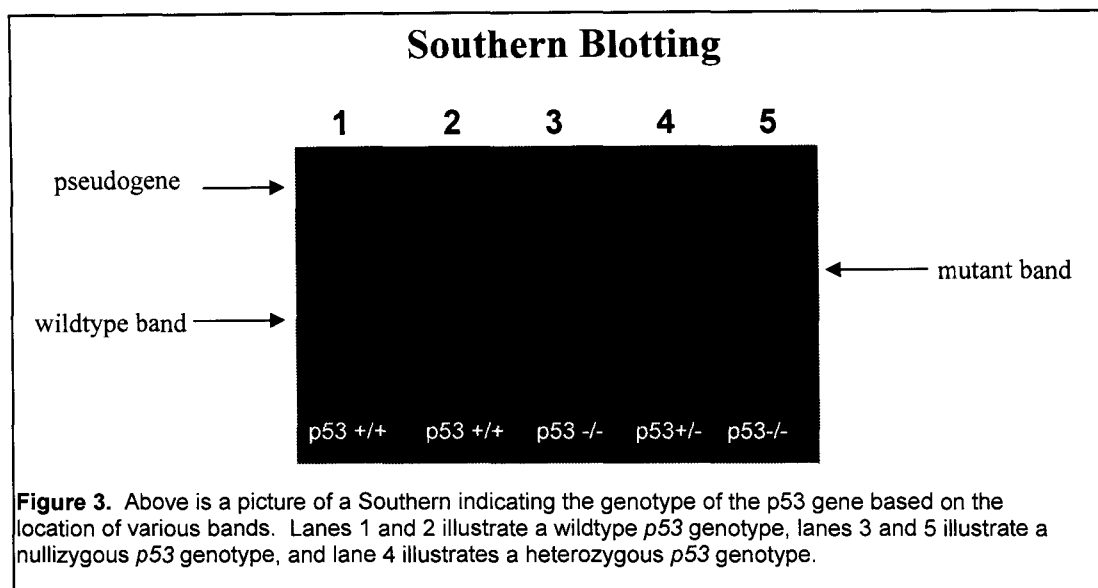
probe overnight; the buffer is then removed and the membrane rinsed in 2X SSC. A series of solutions are then used for washing the membrane for ten minutes per solution (Solution 1: 2X SSC, 0.1% SDS; Solution 2: 0.5X SSC, 0.1% SDS; Solution 3: 0.2X SSC, 0.2% SDS). Once the radioactivity of the membrane is between 2,000 and 3,000 cpm, the membrane is exposed on x-ray film overnight at -80°C. The radio-labeled probe is created using 100ng of DNA specific to Exons four through six of the *p53* gene.

**Balb/C Genotyping:** To characterize a mouse as having a BALB/c background or being pure BALB/c, a version of PCR specified by Yu et al was used. The primers were labeled Balbf and Balbr for forward and reverse primers, respectively. The primers are specific for the *prkdc* gene and are targeted to analyze the presence of the *prkdc* polymorphism seen in BALB/c mice. The polymorphism shortens the amplified band targeted by the above primers from approximately 500bp to 250bp—this is analyzed in the final photograph of the agarose gel. The PCR reaction mix is the same as for BubR1 (with the exception of the primers). The PCR conditions are as follows: 94°C for 30 seconds, followed by 49°C for 30 seconds, then 72°C for 30 seconds and this is cycled 40 times. There is then a final extension at 70°C for ten minutes. The PCR product is then

digested by the enzyme BsmB1 in a fashion similar to that used in Southern blotting. This digest is incubated at 55°C for 3 hours. The product is then analyzed by gel electrophoresis on a 2% agarose gel.

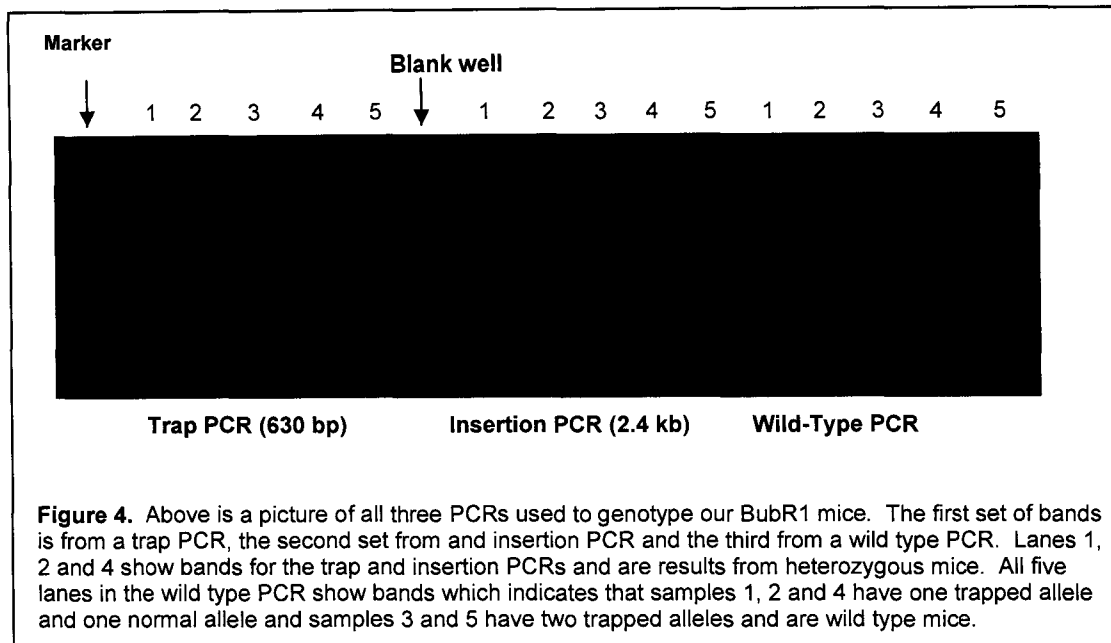
### **Results**

**P53 Genotyping:** Our p53 BMR probe is made specifically for mice bred in a p53 BMR background. The radio-labeled probe, when added to the membrane as mentioned above, binds to the p53 sequence wild type and mutant allele. The probe also binds a pseudogene regardless of its binding to the wild type and/or mutant allele. The binding to the pseudogene proves that the probe properly functioned and the DNA concentration was adequate for binding. Once the membrane is radio-labeled, washed, and the film is exposed, the resulting bands can be analyzed to determine the genotype of the specimen.



BubR1 characterization: The PCR reactions for BubR1 genotyping (as described in Materials and Methods) are run on a 1% agarose gel. The gel is then photographed and can be analyzed in a similar fashion to the p53 membrane to determine genotype. Bands that appear during reactions for the trap and insertion PCRs indicate the presence of an interrupted BubR1 gene and are characteristic of heterozygous mice. Bands seen in the wild type PCR reaction are indicative of a wild type, or normal, BubR1 allele and are characteristic of wild type mice. If no band is seen in the three PCRs, the mouse can be assumed to

have lost its wild copy of BubR1.



**Backcrossing into BALB/c:** Initially, we began genotyping our mice in the BALB/c background using the PCR reaction described in Materials and Methods. However, we have run into difficulties with the PCR, as it produces several nonspecific bands, and we have reason to believe the results generated by Yu et al were incorrect or mislabeled. As a result, we began to backcross our BALB/c colony with a goal of eventually reaching an almost pure BALB/c background (considered to be N10).

So far, we have performed a total of three backcrosses (N3) to place our p53/BubR1 mice into a BALB/c background. With three backcrosses, we achieve an 87.5% BALB/c background. Our goal is to backcross our colony seven more

times to approach what is scientifically considered a pure BALB/c background. So far, we have not yet seen tumorigenesis occurring in the mammary tissue of our females, but we will continue monitoring their tumor susceptibility.

# **Intercrosses:**

**Table 1.** Results from intercrosses of BubR1/p53 double mutants in the BALB/c background.

## **BubR1+/- p53+/- X BubR1+/- p53+/- [n=1]**

BubR1 WT/p53 WT	BubR1 WT/p53 het	BubR1 WT/ p53 null	BubR1 het/ p53 WT	BubR1 het/p53 het	BubR1 het/ p53 null	BubR1 null/ p53 null
1	0	1	0	4	1	0

## **BubR1+/- p53+/- X BubR1+/+ p53 +/- [n=3]**

BubR1 WT/p53 WT	BubR1 WT/p53 het	BubR1 WT/ p53 null	BubR1 het/ p53 WT	BubR1 het/p53 het	BubR1 het/ p53 null
2	3	1	3	4	4

## **BubR1+/- p53-/- X BubR1+/+ p53+/- [n=1]**

BubR1 WT/p53 WT	BubR1 WT/p53 het	BubR1 WT/ p53 null	BubR1 het/ p53 WT	BubR1 het/p53 het	BubR1 het/ p53 null
0	0	3	0	2	0

These tables show the genotypes of the crossed mice as well as the number [n] of each type of intercross. The tables contain data for each possible genotype for the offspring

of these crosses. Mice were genotyped using the PCR and Southern strategies described in Materials and Methods.

**Tumor Formation:** So far, we have sacrificed several mice with some form of an abnormality in one or many of their organs. As mentioned before, we have yet to observe a cancer phenotype in mammary tissue, but we have seen abnormalities in the colon of a few mice that could be indicative of cancer formation. This is not surprising as BubR1 levels are often mutated in human colon cancers. The tumor types have yet to be determined by a pathologist, but the summary of the tumor or cancerous locations can be seen below.

<b>Tumor Location</b>	<b>Number of Cases</b>
spleen	2
thymus	3
Colon	2
other	3

**Table 2.** This table shows the numbers of abnormal or cancerous tissues seen in sacrificed mice and indicates their specific locations.

#### **Future Work**

In the future we plan to backcross our BALB/c colony seven more times (N10 generation) to obtain the mutants in a pure BALB/c background. We will also determine if the pure BALB/c background and p53 deficiency will allow us to rescue the BubR1 embryonic lethality observed between 6.5 and 8.5dpc. Until then, we plan to continue monitoring our

colony for any novel phenotypes. As mentioned before, we have preliminary data that suggest increased tumor susceptibility in the colons of these double mutant BALB/c mice. Many mice have been sacrificed and determined to have several colon polyps which will be confirmed by a pathologist.

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